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Plantaricins S and T, Two New Bacteriocins Produced by *Lactobacillus plantarum* LPCO10 Isolated from a Green Olive Fermentation

R. JIMÉNEZ-DÍAZ,[1]†* R. M. RIOS-SÁNCHEZ,[2] M. DESMAZEAUD,[1] J. L. RUIZ-BARBA,[3] AND J.-C. PIARD[1]

Station de Recherches Laitières, Institut National de la Recherche Agronomique-CRJ, 78352 Jouy-en-Josas Cedex, France,[1] and Departamento de Microbiología, Facultad de Biología,[2] and Instituto de la Grasa y sus Derivados (C.S.I.C.), U.E.I. de Biotecnología de Alimentos,[3] 41012 Seville, Spain

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Twenty-six strains of *Lactobacillus plantarum* isolated from green olive fermentations were tested for cross-antagonistic activities in an agar drop diffusion test. Cell-free supernatants from four of these strains were shown to inhibit the growth of at least one of the *L. plantarum* indicator strains. *L. plantarum* LPCO10 provided the broadest spectrum of activity and was selected for further studies. The inhibitory compound from this strain was active against some gram-positive bacteria, including clostridia and propionibacteria as well as natural competitors of *L. plantarum* in olive fermentation brines. In contrast, no activity against gram-negative bacteria was detected. Inhibition due to the effect of organic acids, hydrogen peroxide, or bacteriophages was excluded. Since the inhibitory activity of the active supernatant was lost after treatment with various proteolytic enzymes, this substance could be classified as a bacteriocin, designated plantaricin S. Plantaricin S was also sensitive to glycolytic and lipolytic enzymes, suggesting that it was a glycolipoprotein. It exhibited a bactericidal and nonbacteriolytic mode of action against indicator cells. This bacteriocin was heat stable (60 min at 100°C), active in a pH range of 3.0 to 7.0, and also stable in crude culture supernatants during storage. Ultrafiltration studies indicated that plantaricin S occurred as multimolecular aggregates and that the size of the smallest active form is between 3 and 10 kDa. In sodium dodecyl sulfate-polyacrylamide gels, plantaricin S migrated as a peptide of ca. 2.5 kDa. Maximum production of plantaricin S was obtained in a fermentor system in unregulated pH and log-phase cultures of *L. plantarum* LPCO10 in MRS broth plus 4% NaCl. In these culture conditions, a second bacteriocin (designated plantaricin T) was produced in late-stationary-phase cultures of *L. plantarum* LPCO10. On the basis of its biological activity, its sensitivity to various enzymes, and its molecular weight (lower than that of plantaricin S) as assessed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, plantaricin T appeared different from plantaricin S. Curing experiments with *L. plantarum* LPCO10 resulted in the appearance of variants that no longer produced either of the two bacteriocins but that were still immune to both of them.

Lactobacillus plantarum is a saprophyte often associated with plant and fermenting materials (6). In food and feed fermentations (such as vegetables [10], sausage products [2], and silages [4]), it plays a major role in the preservation of the fermented products. In some cases, *L. plantarum* has been used as a starter culture, but in most processes lactic acid fermentations rely on bacteria which are naturally present.

Production of Spanish-style green olives is a traditional lactic acid fermentation based on an empirical process in that the fruits are handled in order to favor the development of *L. plantarum* in the fermentation brines. By fermenting the sugars contained in the fruits, *L. plantarum* provides the high quantities of lactic acid necessary for olive preservation (9). However, the competing natural flora present in the fruits often predominate over *L. plantarum*, inhibiting its growth in the brines. In that case, lactic acid is not present in the large amounts needed for the adequate preservation of olives, and spoilage of fruits occurs through subsequent contamination by other organisms (9). Technological improvements are necessary to accelerate the lactic acid fermentation of olives by *L. plantarum*; the use of starter cultures containing modified *L. plantarum* strains which can produce, for example, bacteriocins has been suggested by Daeschel and Flemming (6) for other vegetable fermentations. In this regard, bacteriocin-producing *L. plantarum* strains will be useful not only to allow *L. plantarum* to predominate over the competing natural flora in brines but also to help in the preservation of olives against spoilage bacteria.

Bacteriocins are bacterial proteins or peptides that show a bactericidal mode of action against closely related species (33). These substances are of particular interest as they are proteinaceous and may thus be degraded during digestion in humans and other animals. Many of the lactic acid bacteria (LAB) produce bacteriocins (15–17, 24). In *L. plantarum*, two bacteriocins have been partially characterized: plantaricin A, produced by *L. plantarum* C-11 isolated from a cucumber fermentation (7); and plantacin B, from *L. plantarum* NCDO 1193 (37). However, nothing is known about bacteriocin production by *L. plantarum* strains involved in olive fermentations. In an attempt to fill this gap, we describe the production of plantaricins S and T by *L.*

* Corresponding author.
† Present address: Instituto de la Grasa y sus Derivados (C.S.I.C.), U.E.I. de Biotecnología de Alimentos, Avda. Padre García Tejero, 4, Apdo. 1078, 41012 Seville, Spain.

TABLE 1. Inhibitory spectrum of plantaricin S against competitors of *L. plantarum* or spoilage bacteria in green olive fermentations and against pathogens[a]

Indicator species	No. of strains inhibited/no. tested
Gram-positive bacteria	
Competitor	
Lactobacillus delbrueckii	2/2
L. fermentum	3/4
Lactococcus lactis	
subsp. *cremoris*	9/9
subsp. *lactis*	2/2
Leuconostoc mesenteroides	
subsp. *dextranicum*	1/4
subsp. *mesenteroides*	1/4
Leuconostoc paramesenteroides	2/2
Micrococcus sp.	3/4
Pediococcus sp.	2/4
Pediococcus pentosaceus	1/2
Streptococcus thermophilus	1/2
Spoilage or pathogenic	
Bacillus sp.	0/2
Bacillus cereus	0/1
B. subtilis	0/1
Clostridium sporogenes	0/1
C. tyrobutyricum	2/2
Enterococcus faecalis	6/6
Listeria monocytogenes	0/8
Propionibacterium sp.	2/2
Propionibacterium acidipropionici	0/1
Staphylococcus carnosus	0/1
Other	
Lactobacillus acidophilus	0/1
L. casei	0/1
L. curvatus	1/1
L. helveticus	1/1
L. plantarum	13/26
L. reuteri	0/1
L. sake	1/1
Listeria innocua	0/9
Gram-negative bacteria	
Alcaligenes sp.	0/5
Escherichia coli	0/5
Klebsiella sp.	0/3
Pseudomonas sp.	0/4

[a] Except for *L. plantarum* strains belonging to our strain collection, all other bacteria were obtained from the CNRZ Culture Collection, Jouy-en-Josas, France.

plantarum LPCO10 and the preliminary characterization of these bacteriocins.

MATERIALS AND METHODS

Bacterial strains and cultures. The 26 strains of *L. plantarum* used in this study were isolated from Spanish-style green olive fermentations and have been identified previously (28). They were maintained as frozen stocks at −20°C in distilled water plus 20% (vol/vol) glycerol and propagated twice in MRS medium (Oxoid; Unipath Ltd., Basingstoke, Hampshire, England) before use. The bacterial strains used as indicator organisms are listed in Table 1. All LAB used were cultivated in MRS medium. *Bacillus*, *Enterococcus*, *Listeria*, *Micrococcus*, and *Staphylococcus* strains were grown in brain heart infusion medium (Oxoid). Clostridia were cultivated in RCM medium (Difco Laboratories, Detroit, Mich.), and *Propionibacterium* strains were cultivated in Yeast-Glucose Lemco medium (5); for gram-negative bacteria, tryptic soy broth medium (Oxoid) was used.

Screening cultures for inhibitory compound production and sensitivity. The agar drop diffusion test was performed as follows: strains of *L. plantarum* were grown overnight in MRS broth at 30°C. Culture supernatants were adjusted to pH 7.0 and filtered through a 0.22-μm-pore-size Millex-GV filter (Millipore Corp.). Lawns of each strain were prepared by inoculating ca. 10^6 cells in 4 ml of soft-overlay MRS (0.7% agar) which was poured onto the surface of MRS agar plates (1.5% agar); the plates were then allowed to dry for 15 min in a drying hood. For *Clostridium* and *Propionibacterium* species, the agar plates were preincubated anaerobically for 4 to 6 h before being overlaid with the soft agar to reduce the dissolved oxygen in the culture medium. Ten microliters of the supernatants was spotted onto the indicator lawns, and then the plates were allowed to dry for 10 min. The plates were incubated at 30°C for 1 to 4 days, depending on the growth of the indicator strain, and they were examined for inhibition zones. Tests for the presence of bacteriophage were carried out by the method of Tagg et al. (33).

For a semiquantitative assay of the bacteriocins, twofold serial dilutions of the supernatant were used, as described previously (3). The indicator strains used were *L. plantarum* 128/2 and *L. fermentum* ATCC 14933. The titer of bacteriocin activity was defined as the reciprocal of the lowest dilution which did not show inhibition of the indicator strain and was expressed in activity units (AU) per milliliter.

Sensitivity to heat and enzymes of the antimicrobial compounds produced by *L. plantarum* LPCO10. Supernatants from log phase (ca. 10 h) and late stationary phase (ca. 40 h) were obtained in a fermentor system in unregulated pH cultures of *L. plantarum* LPCO10 in MRS broth plus 4% NaCl. The supernatants were adjusted to various pH values and heated to 100°C. Samples were removed at different time intervals, and the pH was adjusted to 7.0 prior to bacteriocin titer determination.

To test for enzyme sensitivity, log-phase and late-stationary-phase *L. plantarum* LPCO10 MRS culture supernatants, each containing 51,200 AU of the antimicrobial compound per ml, were treated with dextranase, α-amylase, lipase A, phospholipase C, α-chymotrypsin, ficin, pronase E, proteinase K, thermolysin, trypsin, or subtilopeptidase A, at final concentrations of 0.1 mg/ml. Buffers used were as recommended by the supplier (Sigma Chemical Co., St. Louis, Mo.). To prevent bacteriocin inactivation caused by possible protease contaminants in glycolytic and lipolytic enzyme solutions, these were added with protease inhibitors, including benzamidine (final concentration, 5 mM), chymostatin, leupeptin, antipain, and pepstatin A (final concentration, 1 μg/ml each), aprotinin (final concentration, 10 μg/ml), and phenylmethylsulfonyl fluoride (final concentration, 1 mM). Samples were incubated at 37°C for 1 h, and the residual activity was determined. Controls were buffers, heat-inactivated enzymes, or protease inhibitors. To ensure that no proteolytic activity remained in glycolytic and lipolytic enzyme solutions added with the proteinase inhibitors, these solutions were tested against bovine serum albumin, and the absence of proteolysis was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

To exclude potential inhibition by hydrogen peroxide, culture supernatants were treated with catalase (Sigma) at a final concentration of 100 U/ml as described earlier (23). They were maintained at 25°C for 35 min, and their titers were determined.

Mode of action of the bacteriocin plantaricin S, produced by *L. plantarum* LPCO10. Cell-free, filter-sterilized, log-phase *L. plantarum* LPCO10 MRS culture supernatants (9.9 ml) containing 3,200 AU/ml were adjusted to pH 3.0, 4.0, 5.0, 6.0, or 7.0. A log-phase *L. plantarum* 128/2 (bacteriocin indicator strain) culture in MRS broth was centrifuged (6,000 × *g*, 10 min), washed in saline (0.9% NaCl, wt/vol), and resuspended in MRS broth. A fraction (0.1 ml) of this suspension containing about 10^8 CFU/ml was added to the active supernatants, and the mixture was then incubated at 30°C. At different time intervals, the optical density at 590 nm was measured, and the number of CFU per milliliter was determined after sample treatment with pronase E (0.1 mg/ml) for 1 h at 37°C to inactivate residual bacteriocin. A similar experiment was performed at pH 7.0 with log- and stationary-phase *L. plantarum* 128/2 cultures. In one control, *L. plantarum* 128/2 was inoculated into MRS spent broth derived from bacteriocin-deficient derivative *L. plantarum* 55-1 cultures adjusted to pH 3.0 to 7.0; in another control, *L. plantarum* 128/2r, a spontaneous bacteriocin-resistant derivative from *L. plantarum* 128/2, was used in place of the sensitive strain.

For dose-response effect studies, ammonium sulfate (50% saturated)-precipitated plantaricin S was added at various dilutions to log-phase cell suspensions of *L. plantarum* 128/2 (ca. 2×10^7 CFU/ml) in sodium phosphate buffer (50 mM; pH 7.0). After 30 min of incubation, samples were removed and the number of CFU per milliliter was determined as described above. The "hit" killing kinetics for plantaricin S-induced cell death was then calculated by extrapolation from the experimental data, as described previously (32). *L. plantarum* 128/2 cells which survived after 30 min of incubation in 300 to 1,000 AU of plantaricin S per ml were isolated, and their resistance to the bacteriocin was tested.

Production studies. MRS broth with or without NaCl (4%, wt/vol; 1.5 liters) was sterilized by autoclaving, aseptically transferred to a fermentor bowl connected to an automatic pH controller (Fermentor Set 2; SGI, Toulouse, France), and then inoculated with 2% (vol/vol) of an overnight culture of *L. plantarum* LPCO10. For production studies at controlled pH, the initial pH of the MRS broth was adjusted to 4.0, 5.0, 6.0, or 7.0 and maintained at this value during the fermentation, using 2 M sodium hydroxide. The temperature was held at 30°C, and agitation was set at 80 rpm. Samples were removed at different time intervals for the determination of both optical density at 590 nm and antibacterial activity.

Stability of plantaricin S at different temperatures and pHs. Active culture supernatants from log-phase *L. plantarum* LPCO10 MRS cultures were adjusted to various pH values and sterilized either with a Millex-GV filter or by heat treatment at 100°C for 10 min in a boiling-water bath. The treated aliquots were stored at −20°C, 4°C, or room temperature. At different time intervals, aliquots were removed to determine inhibitory activity.

Ammonium sulfate precipitation of plantaricin S. Cell-free supernatants from log-phase *L. plantarum* LPCO10 MRS cultures at various pH values were treated with solid ammonium sulfate to 0, 30, 33, 35, 40, 50, or 60% saturation. The mixtures were stirred for 2 h at 4°C and centrifuged at 20,000 × *g* for 1 h (4°C). The precipitates were resuspended to the initial volume with sodium phosphate buffer (50 mM; pH 7.0), and the bacteriocin titer was determined in both the precipitate and the supernatant.

Ultrafiltration studies on plantaricin S. The size of plantaricin S was estimated from extracts of a log-phase *L. plantarum* LPCO10 MRS culture. The plantaricin was prepurified with 50% saturated ammonium sulfate and resuspended to 1/30 volume in sodium phosphate buffer (50 mM; pH 7.0). Several aliquots (1 ml) were ultrafiltered through various Filtron membranes (Filtron Technology Corp., Northborough, Mass.), including 1,000-, 300-, 100-, 30-, 10-, and 3-kDa molecular exclusion sizes. Antibacterial activity was determined in retained and flowthrough fractions.

SDS-PAGE. Comparative SDS-PAGE analysis of plantaricin S and plantaricin T was performed as follows: active extracts were prepared by addition of 50% saturated ammonium sulfate to the supernatants from log-phase (plantaricin S) and late-stationary-phase (plantaricin T) cultures. The resulting pellets were dissolved in 1/30 volume of sodium phosphate buffer (50 mM; pH 7.0). The active extracts were desalted, using a PD-10 column (Pharmacia LKB Technology, Uppsala, Sweden) and sodium phosphate (50 mM; pH 7.0) as a running buffer. SDS-PAGE was performed as described previously (30), but a 10-cm layer of 18.0% instead of 16.5% acrylamide resolving gel was used. After electrophoresis, the gel was divided; one part was silver stained (19), and the other part was used for detection of antimicrobial activity as described previously (5).

Plasmid curing experiments. Novobiocin (0.125 to 0.5 μg/ml) was used to treat *L. plantarum* LPCO10 cultures, as described previously (27). Cultures were then plated onto MRS agar plates to obtain ca. 10 to 20 colonies per plate. After 18 h at 30°C, MRS soft agar containing *L. plantarum* 128/2 or *L. fermentum* ATCC 14933 as the indicators strain was poured onto the plates, which were incubated for an additional 24 h at 30°C. Colonies without clear zones of inhibition were purified on MRS agar, repeatedly transferred into MRS broth with or without NaCl (4%, wt/vol), and checked for inability to inhibit growth of *L. plantarum* 128/2 and *L. fermentum* ATCC 14933 strains.

The immunity of the nonproducer variants to the antimicrobial substances was examined by spotting active *L. plantarum* LPCO10 supernatants obtained from MRS broth with or without NaCl on lawns of these derivatives.

Plasmid isolation. Plasmid DNA in *L. plantarum* LPCO10 producer and nonproducer variants was isolated (1) and analyzed (27) as described previously. The parental *L. plantarum* LPCO10 strain was used as a source of plasmid markers (27).

RESULTS

Screening for inhibitory compounds and spectrum of activity. Twenty-six *L. plantarum* strains isolated from green olive fermentations were screened for antimicrobial production and sensitivity, using an agar drop diffusion test. Four of these strains were found to produce an antibacterial compound with a variable spectrum of activity against indicators. The largest spectrum of inhibition was shown by *L. plantarum* LPCO10, which inhibited 13 of the 26 indicator strains. This strain was therefore selected for further studies. *L. plantarum* LPCO10 inhibitory activity was directed against several natural competitors of *L. plantarum* in olive fermentation, including taxonomically related and nonrelated bacteria (Table 1). Among gram-positive olive spoilage organisms, the antimicrobial compound showed activity against *Propionibacterium* sp. and *Clostridium tyrobutyricum*. In addition, *Enterococcus faecalis*, a frequent contaminant in olive fermentation, was highly sensitive to the antibacterial substance. However, the spoilage bacilli were not inhibited and neither were the pathogenic strains of

Listeria monocytogenes. Also, no action was observed against spoilage and pathogenic gram-negative bacteria.

The observed inhibition was not due to low pH since the active supernatant was adjusted at pH 7.0 before use. Treatment of active supernatants with catalase did not modify the initial inhibitory titer, indicating that hydrogen peroxide did not account for the observed inhibition. Supernatant dilution until extinction, which showed diminishing zones of inhibition but no plaques, excluded the possibility of bacteriophage infection.

Heat and enzyme sensitivity of plantaricin S, produced by *L. plantarum* LPCO10. The inhibitory activity observed in log-phase cultures of *L. plantarum* LPCO10 was 100% stable to heat treatment (100°C) for up to 60 min at pH 4.0, 6.0, and 7.0 (not shown). However, at pH 5.0, 50% of the activity was destroyed following a 10-min heat treatment. The chemical nature of the active substance was assessed with different enzymes. With the exception of lysozyme, treatment of active supernatants containing 51,200 AU of the antimicrobial substance per ml with various glycolytic (α-amylase and dextranase), lipolytic (lipase A and phospholipase C), and proteolytic (α-chymotrypsin, trypsin, ficin, pronase E, proteinase K, thermolysin, and subtilopeptidase A) enzymes led to total inactivation of antibacterial activity (not shown). These results suggest that the antibacterial compound produced by *L. plantarum* LPCO10 in the log phase of growth has a proteinaceous nature and that lipidic and glucidic moieties are required for biological activity. On the basis of the definition of bacteriocins of gram-positive bacteria given by Tagg et al. (33) and Klaenhammer (15) and by using the current properties generally observed in bacteriocins from LAB (17, 24), the inhibitor produced by *L. plantarum* LPCO10 in the log phase of growth can be classified as a bacteriocin, which we designated plantaricin S.

Bactericidal action of plantaricin S. To characterize the mode of action of plantaricin S, both viability and lysis of *L. plantarum* 128/2 were monitored in the presence of bacteriocin. A rapid decline in viability of this strain was observed in the first minutes of contact with plantaricin S at all pH values (Fig. 1). However, the sharpest decline was exhibited at pH 3.0 and 4.0, indicating that the optimum activity pH of plantaricin S was in this range. The optical density at 590 nm did not change at any pH during the 22 h of incubation, indicating that no lysis of the indicator occurred. Therefore, plantaricin S appears to be a bactericidal, nonbacteriolytic bacteriocin.

To examine whether the antimicrobial activity of plantaricin S depends on the physiological state of the indicator strain, survival of both log- and stationary-phase *L. plantarum* 128/2 cultures in contact with plantaricin S was monitored. In log-phase sensitive cells, a 3-log reduction in viability was observed after 30 min of incubation, and this decline reached 4 logs within 3 h (Fig. 2). In contrast, stationary-phase sensitive cells were unaffected by plantaricin S. The spontaneous *L. plantarum* 128/2-resistant derivative was not affected when it was incubated in the presence of plantaricin S in any phase of growth.

An indicator *L. plantarum* 128/2 strain was treated for 30 min with various doses of plantaricin S (Fig. 3). Extrapolation from the experimental data reveals 1.9, 1.0, 0.65, and 0.6 hit killing kinetics for 0 to 30, 30 to 150, 150 to 300, and 300 to 1,000 AU of plantaricin S per ml, respectively (not shown). Interestingly, analysis of cells which survived at high concentrations of plantaricin S revealed a plantaricin S-resistant (PlS^r) *L. plantarum* 128/2 population representing about 1.8% of the initial inoculum.

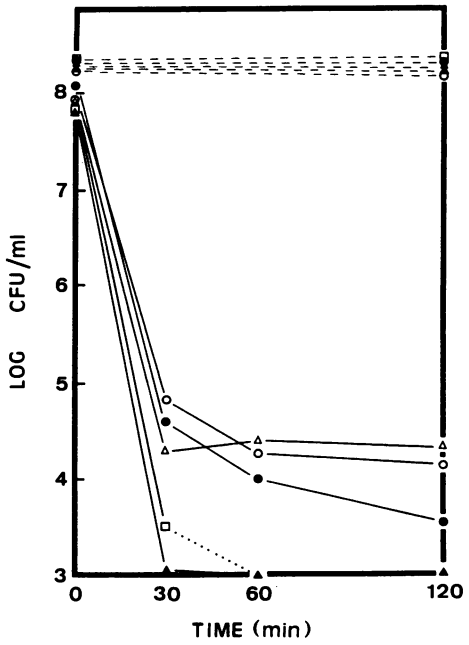

FIG. 1. Bactericidal effect of plantaricin S on a sensitive strain (*L. plantarum* 128/2). Cell-free supernatant from strain LPCO10 containing 3,200 AU of plantaricin S per ml at pH 3.0 (□), pH 4.0 (▲), pH 5.0 (△), pH 6.0 (○), or pH 7.0 (●). Dotted lines with the same symbols represent the controls adjusted to the same pH values, which consisted of MRS spent broth from *L. plantarum* 55-1, a bacteriocin-deficient derivative of *L. plantarum* LPCO10.

Effect of pH, heat treatment, and temperature on stability of plantaricin S after storage. To facilitate further purification, the stability of plantaricin S after storage as a function of pH and temperature was assessed on filter-sterilized or heat-treated active supernatants. At all pH values tested, the activity of the supernatants remained more stable when supernatants were filter sterilized than when they were heat treated (data not shown). Storage of the former at −20°C and at any pH value even increased the activity two- to fourfold within 60 days. These results could indicate dissociation of plantaricin S complexes into their smaller, more active subunits after sample freezing and thawing; the antagonistic properties of these small active subunits would be stronger than those of nondissociated plantaricin S, as observed for other bacteriocins (14, 22, 25).

Ammonium sulfate precipitation of plantaricin S. Plantaricin S was partially purified from culture supernatants with ammonium sulfate. At pH 3.0 to 7.0, 40% saturated ammonium sulfate gave a pellet which contained total initial plantaricin S activity (not shown). Furthermore, in certain conditions (i.e., at pH 3.0 with 60% ammonium sulfate or at pH 4.0 in the range of 40 to 60% saturation), an increase of 200% of the total initial plantaricin S activity was observed. Increases of plantaricin S activity could be explained if ammonium sulfate acts as a dissociating agent (discussed below). Controls consisting of MRS broth treated in the same way did not yield inhibition of indicator strains.

Assessment of plantaricin S molecular weight. Ultrafiltration of a plantaricin S-containing extract, partially purified by ammonium sulfate, showed that all activity was present in the retentate with the 3,000-Da cutoff membrane. When membranes with a higher-molecular-weight cutoff were

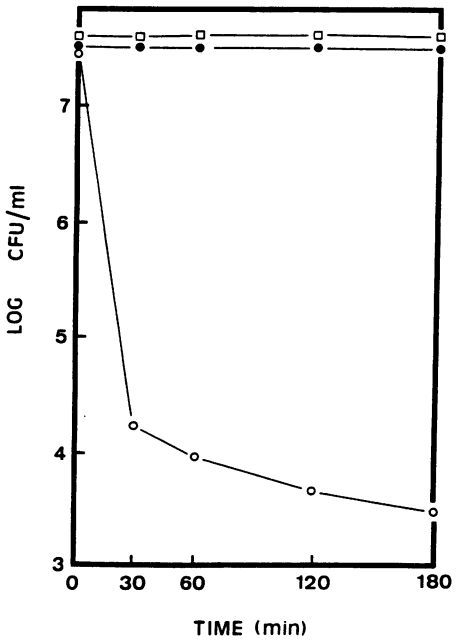

FIG. 2. Effect of plantaricin S on *L. plantarum* 128/2, a plantaricin S-sensitive strain, and on *L. plantarum* 128/2r, a spontaneous plantaricin S-resistant derivative of *L. plantarum* 128/2. *L. plantarum* 128/2 in the log (○) or stationary (●) phase of growth; *L. plantarum* 128/2-resistant derivative in the log or stationary (□) phase of growth.

used, increasing amounts of plantaricin S were recovered in the ultrafiltrate (Table 2). Partial loss of plantaricin S activity was observed during ultrafiltration, possibly due to adsorption of plantaricin S to membranes. These results suggest that the molecular weight of the smallest unit of plantaricin S is in the range of 3,000 to 10,000 and that aggregates of plantaricin S of variable sizes are present in the extract. Indeed, preliminary ultrafiltration studies with crude plantaricin S obtained in MRS cultures of *L. plantarum* LPCO10 without previous ammonium sulfate precipitation showed retention of total plantaricin S activity by Filtron membranes of 300,000-molecular-weight exclusion size (data not shown). Disruption of these high-molecular-weight aggregates, although incomplete, seemed to occur upon treatment with ammonium sulfate.

TABLE 2. Ultrafiltration of plantaricin S

Membrane mol-wt cutoff[a]	AU (% initial plantaricin S activity)[b]	
	Retentate	Ultrafiltrate
1,000,000	2,200 (17.2)	7,300 (57.0)
300,000	3,600 (28.8)	6,500 (50.8)
100,000	7,900 (61.7)	4,900 (38.3)
30,000	7,000 (54.7)	2,900 (22.6)
10,000	10,000 (78.1)	200 (1.56)
3,000	11,300 (88.3)	0 (0.0)

[a] Filtron membranes were used.
[b] 12,800 AU of plantaricin S was subjected to ultrafiltration through each molecular-weight cutoff membrane.

Production studies and evidence of a second bacteriocin produced by *L. plantarum* LPCO10. To monitor production, the most sensitive (*L. fermentum* ATCC 14933) and the least sensitive (*L. plantarum* 128/2) indicator strains were used. Production of plantaricin S was examined in MRS fermentor cultures of *L. plantarum* LPCO10. No plantaricin S activity was observed against *L. fermentum* ATCC 14933 when *L. plantarum* LPCO10 was grown in MRS broth maintained at pH 4.0, and low activity (3,200 AU/ml) was detected in pH 7.0-regulated cultures (not shown). In all other culture conditions tested (Fig. 4), plantaricin S activity reached a peak and subsequently declined. In cultures maintained at

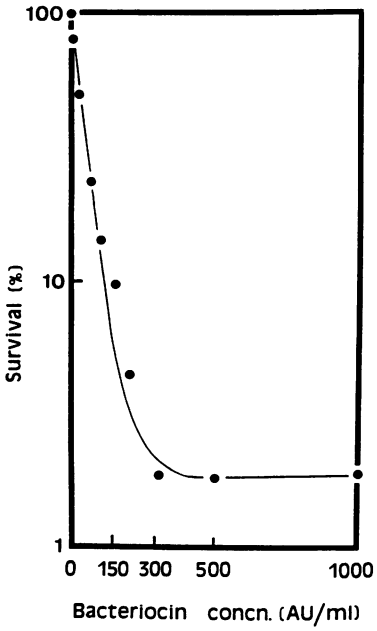

FIG. 3. Survival of *L. plantarum* 128/2 treated for 30 min with various doses of plantaricin S.

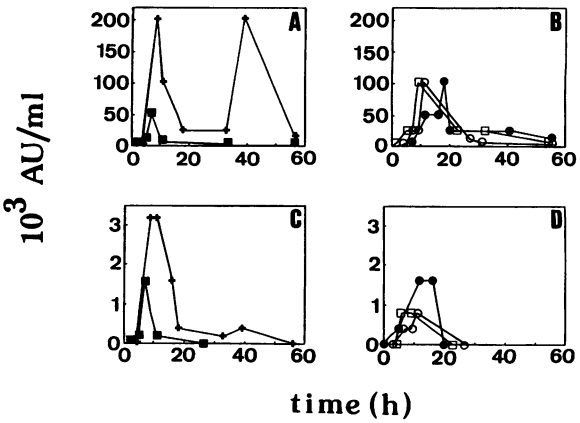

FIG. 4. Production of plantaricins S and T by *L. plantarum* LPCO10 in MRS broth, using a fermentor system. Plantaricins S and T were assayed against *L. fermentum* ATCC 14933 (A and B) and *L. plantarum* 128/2 (C and D). Production of plantaricins S and T in nonregulated-pH cultures in the absence (■) or presence (+) of 4% NaCl. The bacteriocins produced in log phase (first peak) and in late stationary phase (second peak) were designated plantaricins S and T, respectively. Production of plantaricin S at pH regulated 5.0 (○), pH 6.0 (□), or pH 5.0 in the presence of 4% NaCl (●). Note that the scale used for plantaricin S units is different in panels A and B and in panels C and D.

TABLE 3. Comparative inhibitory spectrum of plantaricins S and T against competitors of *L. plantarum* or spoilage bacteria in green olive fermentations

Indicator species	AU/ml	
	Plantaricin S	Plantaricin T
Lactobacillus curvatus	800	0
L. fermentum	3,200	1,600
L. fermentum ATCC 14933	25,600	25,600
L. helveticus	800	400
L. plantarum		
LPS10	1,600	200
LPS5	1,600	200
128/2	1,600	200
LPCO1	1,600	200
L. sake	25,600	6,400
Pediococcus pentosaceus	1,600	400
Lactococcus cremoris	1,600	400
Propionibacterium sp.	1,600	800

pH 5.0 or 6.0, bacteriocin production was lower than given above, even in the presence of 4% NaCl (Fig. 4B and D). In nonregulated pH cultures, the addition of 4% NaCl to MRS broth resulted in a significant increase in plantaricin S activity (Fig. 4A and C). The presence of salt also promoted the appearance of a second antimicrobial activity peak after the producer had entered the stationary phase of growth. This inhibitor (designated plantaricin T), corresponding to the second peak, was highly active against *L. fermentum* ATCC 14993 (Fig. 4A) and was poorly active with the less sensitive *L. plantarum* 128/2 indicator (Fig. 4C).

It is noteworthy that the time course of plantaricin S activity against *L. plantarum* 128/2 did not always parallel that against *L. fermentum* ATCC 14933. For example, MRS broth cultures regulated at pH 5.0 and 6.0 provided a higher titer than non-pH-regulated cultures in MRS broth when *L. fermentum* ATCC 14933 was used as an indicator strain (Fig. 4A and B), whereas the opposite was observed when *L. plantarum* 128/2 was used as the indicator (Fig. 4C and D).

Comparative properties of plantaricins S and T. Plantaricin T exhibited the same heat resistance and the same sensitivity to proteolytic enzymes as plantaricin S. However, in contrast to the latter, plantaricin T was not affected by treatment with α-amylase or lipase A (data not shown). This suggests that the two bacteriocins are chemically different. The two bacteriocins exhibited similar spectra of activity except that only plantaricin S inhibited growth of *L. curvatus* (Tables 1 and 3). Although the two bacteriocins showed the same level of biological activity against the indicator *L. fermentum* ATCC 14933, plantaricin T exhibited a lower level of activity than plantaricin S when tested against various indicators (Table 3).

SDS-PAGE analysis of plantaricins S and T. Further comparative analysis of plantaricins S and T was achieved by SDS-PAGE, and the protein bands corresponding to the bacteriocins were identified by antibacterial activity. A zone of growth inhibition could be detected at a position around 2.5 kDa for plantaricin S, whereas plantaricin T migrates at a slightly lower position (Fig. 5A and B). Extracts originating from plantaricin-deficient derivatives did not show bacteriocin activities in an SDS-polyacrylamide gel (not shown).

Plasmid-curing experiments. After treatment with novobiocin at 0.125 to 0.5 μg/ml, 565 isolated colonies of *L. plantarum* LPCO10 were tested for plantaricin S production in MRS solid medium. Twenty-five (4.4%) colonies failed to induce clear zones of inhibition in lawns of *L. fermentum* ATCC 14933 or *L. plantarum* 128/2 (Fig. 6). However, when the 25 suspected non-plantaricin S producers were purified on MRS agar and then repeatedly transferred into MRS broth with or without 4% NaCl, only 4 of them (0.7%) no longer produced plantaricin S or T. Interestingly, these four plantaricin-deficient derivatives were still immune to both plantaricins S and T.

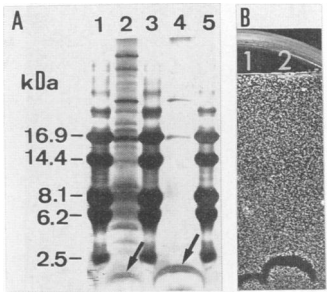

FIG. 5. SDS-PAGE and detection of antimicrobial activity in the gel. (A) Silver-stained gel: lanes 1, 3, and 5, molecular weight standards (size indicated on the left); lane 2, plantaricin T-containing extract; lane 4, plantaricin S-containing extract. (B) Gel fixed in 20% isopropanol–10% acetic acid and washed in deionized water as described in reference 5. The gel was subsequently placed on an MRS agar plate and overlaid with MRS soft-agar medium containing *L. plantarum* 128/2: lane 1, plantaricin T-containing extract; lane 2, plantaricin S-containing extract. Arrows indicate the respective bacteriocin bands.

Plasmid profile analysis of *L. plantarum* LPCO10 and of 52 derivatives isolated after novobiocin treatment (including the 25 mentioned above) showed a 94% curing efficiency of at least one of the plasmids in *L. plantarum* LPCO10. From the numerous plasmids harbored by the parental strain, all except the smallest (2.4 kb) plasmid were randomly lost in the variants. Analysis of the four plantaricin-deficient variants showed that two of them lacked the 27- and 18-kb plasmids and the other two had lost the 49-, 27-, and 18-kb plasmids (Fig. 7). However, these plasmids were also lost in

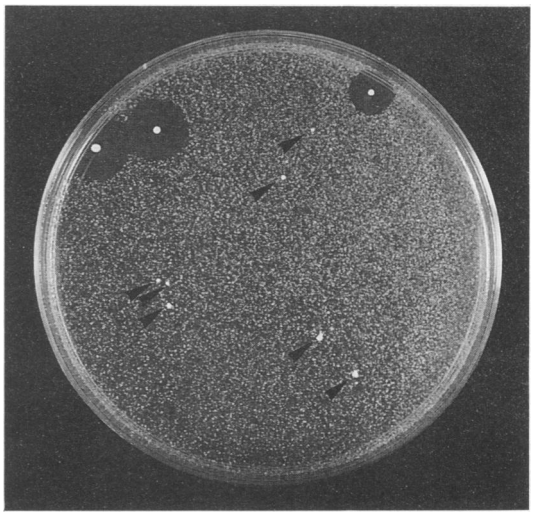

FIG. 6. Presumptive PlS^+ PlT^+ and PlS^- PlT^- colonies isolated after incubation of *L. plantarum* LPCO10 in MRS broth containing 0.125 to 0.5 μg of novobiocin per ml. *L. fermentum* ATCC 14933 was used as the indicator microorganism.

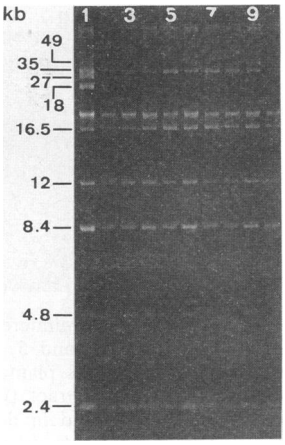

FIG. 7. Agarose gel electrophoresis of plasmid DNA detected in novobiocin-treated variants from *L. plantarum* LPCO10. Lane 1, *L. plantarum* LPCO10; lanes 2, 6, 9, and 10, PIS^+ PIT^+ variants from *L. plantarum* LPCO10 lacking the 27- and 18-kb plasmids; lane 7, PIS^+ PIT^+ variants lacking the 49-, 27-, and 18-kb plasmids; lanes 3 and 4, PIS^- PIT^- variants lacking the 27- and 18-kb plasmids; lanes 5 and 8, PIS^- PIT^- variants lacking the 49-, 27-, and 18-kb plasmids.

other analyzed colonies that had retained both plantaricin S and T production phenotypes. Therefore, the determinants for plantaricin S and T production do not appear to be plasmid encoded.

DISCUSSION

Our studies show that 15% of *L. plantarum* strains isolated from Spanish-style green olive fermentations express inhibitory activity against other bacteria. The *L. plantarum* LPCO10 supernatant exhibited the broadest host range, and its inhibitory activity was shown to be mediated by at least one bacteriocin designated plantaricin S. Optimal plantaricin S production was obtained in MRS media plus 4% NaCl at unregulated pH. *L. plantarum* LPCO10 produced high levels of plantaricin S during log phase and also produced another bacteriocin (plantaricin T) with a different activity spectrum in late stationary phase. Although plantaricin T showed a lower biological activity than plantaricin S, both bacteriocins inhibited some strains of the natural competitors of *L. plantarum* (lactobacilli, leuconostocs, pediococci, micrococci, and *Streptococcus thermophilus*) as well as some olive spoilage bacteria (*C. tyrobutyricum*, *E. faecalis*, and *Propionibacterium* sp.). From the standpoint of their inhibitory spectra, plantaricins S and T appear to take an intermediate position between the lantibiotic nisin, which inhibits most gram-positive bacteria (13), and other narrow-spectrum bacteriocins from LAB (15, 24). Plantaricins S and T differ from both plantaricin A and plantacin B since the former is only active against LAB and the latter is only produced in solid media (7, 37). It may be ecologically significant that plantaricins S and T are best produced in conditions close to those of the natural environment of *L. plantarum*. In Spanish-style green olive fermentations, NaCl (4 to 5%) is added to the brine to inhibit undesirable microorganisms (6, 9). In this saline environment, *L. plantarum* competes with the natural flora for sugars and for other nutrients (6, 9, 10, 28) and may be able to dominate the culture by producing high levels of bacteriocin.

Plantaricin T showed the same sensitivity to proteolytic enzymes as plantaricin S. The two bacteriocins differ, as only plantaricin S is inactivated by lipase and α-amylase, suggesting that lipid and carbohydrate are critical to its biological activity. Other bacteriocins produced by LAB have been shown to contain nonprotein moieties (8, 18, 22, 34). Lactocin 27 from *L. helveticus* and a bacteriocin produced by *L. fermenti* have been shown to contain carbohydrate proportions of 36 and 53%, respectively, the majority of which is mannose (8, 34). The latter also contains a lipid moiety of 21% which remains attached to the bacteriocin after chloroform or ether treatment. More recently, leuconocin S, produced by *Leuconostoc mesenteroides*, has been identified as a glycoprotein poorly detectable after silver staining but strongly detected with Alcian blue (18). However, it is not yet certain whether these carbohydrate and lipid moieties are required for biological activity of bacteriocins or whether purification of these molecules was incomplete. Indeed, lactacin F has been shown to be associated with a lipid moiety which could be removed by reversed-phase high-performance liquid chromatography and which was not a prerequisite for lactacin F activity (22).

A clear distinction between plantaricins S and T was obtained in SDS-PAGE, which showed that plantaricin S was resolved as a 2.5-kDa peptide and that plantaricin T migrated as a lower-molecular-weight peptide. However, the actual size of plantaricin T could not be determined because of an erratic migration of peptides smaller than 2.5 kDa in the separating gel (30). Current purification work will indicate whether these two bacteriocins are structurally distinct or whether plantaricin T is a structural variant of plantaricin S.

Attempts to prepurify the most active bacteriocin, plantaricin S, by precipitation with ammonium sulfate showed that, under certain conditions, the activity recovered in the pellet was higher than that present in the initial supernatant. Increased activity has been observed previously upon purification of bacteriocins of seemingly high molecular weight; dissociation of the complexes appears to be responsible for increased activity (3, 14, 20). For example, lacticin 481 activity increased 450-fold upon ammonium sulfate precipitation (25). The heterogeneous size range of plantaricin S (>300 kDa for the native bacteriocin, 3 to 300 kDa for partially purified material, and 2.5 kDa for the SDS-PAGE-purified molecule) may indicate that the increase in total biological activity could also result from multimer dissociation.

As described for most bacteriocins produced by LAB (15, 16, 24, 33), plantaricin S exhibited a bactericidal mode of action against sensitive cells in the log phase of growth. In contrast, stationary-phase cells were insensitive to plantaricin S. Similar results have been reported for other bacteriocins (15, 16, 24, 25, 33). This is reminiscent of ion-channel-forming bacteriocins, which require the presence of a transmembrane potential or of a pH gradient to be active and which are therefore far more active on log-phase cells than on stationary-phase cells (11, 29). Killing of sensitive cells by plantaricin S occurred within a few minutes after plantaricin S addition, and the threshold dose for plantaricin S activity was 50 AU/ml. It was not possible from the dose-response studies to tell whether plantaricin S acted in a single-hit or a multihit fashion. These difficulties could result from the lack of purity of plantaricin S. Interestingly, the bactericidal effect of plantaricin S was shown in a wide range of pH, 3.0 to 7.0, and plantaricin S was highly stable between pH 3.0 and 7.0. These properties should promote a high bactericidal activity of plantaricin S since the pH of green olives goes

from pH 8.0 when *L. plantarum* starts to grow to pH 3.5 to 4.0 when *L. plantarum* enters the stationary phase.

In lactobacilli, the genetic determinant for bacteriocin and immunity can be either plasmid (20, 21, 31) or chromosome (3, 14) associated. We isolated PlS^- PlT^- strains by using plasmid-curing techniques. However, the PlS^- PlT^- strains remained PlS^r PlT^r. In addition, no assignment could be made between PlS^- PlT^- and the loss of a particular plasmid. Difficulties in characterizing a plasmid-borne determinant may be due to (i) poor extraction and/or resolution of high-molecular-weight plasmids (1) or (ii) an episomal event leading to the integration of DNA into the chromosome (21, 24). In LAB, the genes for bacteriocin production and immunity are generally closely located (35, 36) or the two phenotypes are closely linked (26). In the case of *L. plantarum* LPCO10, the two properties are separable. Similarly, in *Pediococcus acidilactici*, pediocin PA-1-deficient derivatives were still immune to the bacteriocin (12). Although unusual, this independence of Bac^+ and Bac^r phenotypes could be exploited to construct plantaricin-immune strains for use in food fermentations along with a plantaricin producer. Work is now in progress to better characterize plantaricins S and T at the molecular level.

The properties of plantaricins S and T described here appear quite promising for development of consistent Spanish-style green olive fermentations of high quality.

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